Elevated Glucose Changes the Expression of Ionotropic Glutamate Receptor Subunits and Impairs Calcium Homeostasis in Retinal Neural Cells

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PURPOSE. Altered glutamatergic neurotransmission and calcium homeostasis may contribute to retinal cell dysfunction and apoptosis in diabetic retinopathy (DR). The purpose of this study was to determine the effect of high glucose on the protein content of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate glutamate receptor subunits, particularly the GluR2 subunit, because it controls Ca2+-permeability of AMPA receptor-associated channels. The effect of high glucose on the concentration of cytosolic free calcium ([Ca2+]i) was also investigated.

METHODS. The protein content of GluR1, GluR2, GluR6/7, and KA2 subunits was assessed by Western blot. Cobalt staining was used to identify cells containing calcium/cobalt-permeable AMPA receptors. The [Ca2+]i changes evoked by KCl or kainate were recorded by live-cell confocal microscopy in R28 cells and in primary cultures of rat retina, loaded with fluo-4.

RESULTS. In primary cultures, high glucose significantly decreased the protein content of GluR1 and GluR6/7 subunits and increased the protein content of GluR2 and KA2 subunits. High glucose decreased the number of cobalt-positive cells, suggesting a decrease in calcium permeability through AMPA receptor-associated channels. In high-glucose–treated cells, changes in [Ca2+]i were greater than in control cells, and the recovery to basal levels was delayed. However, in the absence of Na+, to prevent the activation of voltage-sensitive calcium channels, the [Ca2+]i changes evoked by kainate in the presence of cyclothiazide, which inhibits AMPA receptor desensitization, were significantly lower in high-glucose–treated cells than in control cultures, further indicating that AMPA receptors were less permeable to calcium. Mannitol, used as an osmotic control, did not cause significant changes compared with the control.

CONCLUSIONS. The results suggest that elevated glucose may alter glutamatergic neurotransmission and calcium homeostasis in the retina, which may have implications for the mechanisms of vision loss in DR. (Invest Ophthalmol Vis Sci. 2006;47: 4130–4137) DOI:10.1167/iovs.06-0085

Diabetic retinopathy (DR) is the leading cause of new cases of blindness in working age adults in Western countries. Although DR is usually considered a vascular disease, recent reports have clearly shown that the retinal neurons are also affected by diabetes (reviewed by Barber).2 In fact, loss of color3,4 and contrast sensitivity5 have been reported as early symptoms of neuronal dysfunction of the retina, occurring within 2 years after the onset of diabetes in humans. More recently, it has been demonstrated that retinal neurons undergo apoptosis in diabetic rats, mice, and humans.6–10 Glutamate is the main excitatory neurotransmitter in the retina, and it is essential for the transmission of the visual signal from the photoreceptors to the bipolar cells and from these to the ganglion cells.11 However, results in several studies suggest that glutamate is involved in the development of DR. The conversion of glutamate to glutamine is impaired in the retinas of diabetic rats,12 and the concentration of glutamate is increased in the retinas and vitreous of patients with proliferative DR.13,14 Moreover, we recently demonstrated that the release of D-aspartate evoked by membrane depolarization is increased in diabetic rat retinas and in retinal cell cultures exposed to high glucose,15 indicating that diabetes can affect the mechanisms of glutamate release. Overall, these results show that the extracellular concentration of glutamate in the retina may be increased by diabetes, leading to an overactivation of glutamate receptors and consequent neuronal dysfunction.

Glutamate receptors can be divided into two distinct groups: the fast-acting ligand-gated ion channels and the slower-acting metabotropic receptors. Based on their physiological, pharmacologic, and molecular properties, the ionotropic glutamate receptors are further subdivided into three groups: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA), and N-methyl-D-aspartate (NMDA) receptors. Whereas the metabotropic receptors are single polypeptides linked to G-proteins, the ionotropic receptors are multimeric, with AMPA receptors being assembled from subunits designated GluR1–4; kainate receptors from subunits GluR5–7, KA1, and KA2; and NMDA receptors from NR1, NR2A-D, and NR3A subunits. The ion channels associated with NMDA receptors are highly permeable to Ca2+, whereas AMPA and kainate receptor-associated channels are usually less permeable to Ca2+. However, certain combinations of subunits increase the Ca2+-permeability of AMPA and kainate receptor-associated channels. For example, AMPA receptors lacking the GluR2 subunit are Ca2+-permeable.16–18

Activation of ionotropic glutamate receptors leads to an increase in cytosolic Ca2+ concentration ([Ca2+]i). If these receptors are overstimulated, the sustained increase in [Ca2+]i can induce cell dysfunction and ultimately lead to cell death.19
Excitotoxicity resulting from overstimulation of glutamate receptors has been implicated in other retinal diseases, including retinitis pigmentosa and glaucoma.\textsuperscript{20,21} In diabetes, cumulative evidence shows that Ca\textsuperscript{2+} homeostasis may be impaired, and defects in Ca\textsuperscript{2+} regulation in several tissues, including the nervous system, have been reported (reviewed by Biessels et al.\textsuperscript{22}). In dorsal horn neurons from streptozotocin (STZ)-induced diabetic rats, the activity of N- and L-voltage-sensitive Ca\textsuperscript{2+} channels is substantially increased.\textsuperscript{23,24} Furthermore, immunoreactivity for the Ca\textsuperscript{2+} binding proteins, parvalbumin, and calbindin, is increased in amacrine and bipolar cells of the retina of diabetic rats, suggesting a response to increases in intracellular Ca\textsuperscript{2+} and raising the possibility that diabetes alters Ca\textsuperscript{2+} homeostasis of neurons in the retina.

This study investigated the effect of elevated concentrations of glucose on the protein content of GluR1, GluR2, GluR6/7, and KA2 subunits and the [Ca\textsuperscript{2+}]\textsubscript{i} changes in retinal neural cell cultures. A cobalt uptake staining technique was used to identify neurons that express calcium/cobalt-permeable AMPA receptor-associated channels, which lack the GluR2 subunit.\textsuperscript{25,26} The effect of high glucose on the function of Ca\textsuperscript{2+}-permeable AMPA receptor-associated channels was also investigated by fluorescent Ca\textsuperscript{2+}-imaging, in the absence of Na\textsuperscript{+} [replaced by L-methyl-D-glucamine (NMG)], which prevents membrane depolarization and therefore the opening of voltage-sensitive calcium channels. The cells were stimulated with kainate to cause sustained currents, potentiated by the drug cyclothiazide (CTZ), which inhibits AMPA receptor desensitization.\textsuperscript{27,28} Because NMDA receptors were blocked by Mg\textsuperscript{2+} in the medium, the stimulation with kainate in non-sensitizing conditions allowed Ca\textsuperscript{2+} influx only through Ca\textsuperscript{2+}-permeable receptor-associated channels. Overall, the data demonstrate that an elevated concentration of glucose alters the glutamate receptor subunit content and Ca\textsuperscript{2+} homeostasis in retinal neurons, which could account for some of the functional changes observed in DR.

**METHODS**

**Materials**

Fetal bovine serum was purchased from BioWhittaker (Walkersville, MD) and newborn calf serum was purchased from HyClone (Logan, UT). Trypsin (USP grade) was purchased from Invitrogen Corp. (Carlsbad, CA). Kainate (KA), cyclothiazide (CTZ), and 4-methylglutamate were purchased from Tocris (Ellisville, MO). LY 303070 was a kind gift from Gail Seigel (Ross Eye Institute, SUNY at Buffalo, NY).\textsuperscript{30,31} It has been shown that R28 cells grown with laminin and cAMP have a neuronlike phenotype.\textsuperscript{32} The cells were plated at a seeding density of 1.28 \times 10^5 cells/cm\textsuperscript{2}, in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 mM glucose, supplemented with 10% newborn calf serum and 250 \muM CAMP, on laminin-coated (1 mg/mL) glass-bottomed culture dishes. The cells were maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/air. For experiments, cells were incubated with 15 mM D-glucose yielding a total 20 mM glucose, or for osmotic control, with 15 mM mannitol (plus 5 mM glucose), and cultures were kept for 48 to 72 hours. The concentration of glucose in the control condition was 5 mM.

**Western Blot Analysis of AMPA and Kainate Receptor Subunits**

Cells were washed twice with cold phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na\textsubscript{2}HPO\textsubscript{4}, and 1.8 KH\textsubscript{2}PO\textsubscript{4}, |pH 7.4|) and lysed in 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS supplemented with complete miniprotease inhibitor cocktail tablets. The protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA), and 15 \muM protein (when blotting for GluR1 and GluR6/7) or 60 \muM protein (when blotting for GluR2 and KA2) from each sample were used for immunoblots, after adding 6× concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M diethiothreitol (DTT), 0.012% bromophenol blue) and heating the samples for 5 minutes at 95°C. Proteins were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrothermally to polyvinylidene (PVDF) membranes. The membranes were blocked for 1 hour at room temperature in Tris-buffered saline (in mM: 137 NaCl and 20 Tris-HCl (pH 7.6)) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies (anti-GluR1, 1:500; anti-GluR2, 1:500; anti-GluR6/7, 1:500; and anti-KA2, 1:600) was performed overnight at 4°C. After washing for 1 hour in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 hour at room temperature with an alkaline phosphatase-linked secondary antibody (anti-rabbit IgG 1:20,000 in TBS-T with 1% low fat milk). The membranes were processed for detection of ionotropic glutamate receptor subunits using the ECF system on a gel imager (Versa Doc Imaging System; Bio-Rad), and digital quantification was performed (Quantity One; Bio-Rad).

**Cobalt Staining**

Cobalt staining was performed as described previously,\textsuperscript{16,33} with some modifications. The cells were washed twice with HEPES buffer (in mM: \textit{Ca}\textsuperscript{2+}-free Hanks’ balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH\textsubscript{2}PO\textsubscript{4}, 0.34 Na\textsubscript{2}HPO\textsubscript{4}, 4 NaHCO\textsubscript{3}, and 5 glucose (pH 7.4)) with a dissection microscope. The retinas were digested with 0.1% trypsin (wt/vol) for 15 minutes at 37°C. After digestion, the cells were collected by centrifugation (1 minute, 140 g) and resuspended in Eagle’s minimum essential medium (MEM), containing 5 mM glucose supplemented with 26 mM NaHCO\textsubscript{3}, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 \mug/mL). The cells were plated at a density of 2 \times 10^5 cells/cm\textsuperscript{2} on poly-L-lysine substrate (0.1 mg/mL). For immunoblot analysis, cells were plated on 60-mm plastic Petri dishes. For immunohistochemistry, cells were plated on glass coverslips. For Ca\textsuperscript{2+} imaging studies, cells were plated on 35-mm glass-bottomed culture dishes (MatTek; Ashland, MA). The cells were maintained at 37°C in a humidified incubator with 5% CO\textsubscript{2}/air. After 2 days in culture, cells were incubated with 25 mM D-glucose, yielding a total 30 mM glucose. For osmotic control, cells were incubated with 25 mM mannitol (plus 5 mM glucose in the culture medium) and maintained for a further 7 days. The concentration of glucose in the control condition was 5 mM.

**R28 Cell Culture**

The R28 cell line originated from a mixed population of retinal cells transfected with E1A.NR.3, followed by selection of neuronlike clones, was a generous gift from Gail Seigel (Ross Eye Institute, SUNY at Buffalo, NY).\textsuperscript{30,31} It has been shown that R28 cells grown with laminin and cAMP have a neuronlike phenotype.\textsuperscript{32} The cells were plated at a seeding density of 1.28 \times 10^5 cells/cm\textsuperscript{2}, in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 mM glucose, supplemented with 10% newborn calf serum and 250 \muM CAMP, on laminin-coated (1 mg/mL) glass-bottomed culture dishes. The cells were maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/air. For experiments, cells were incubated with 15 mM D-glucose yielding a total 20 mM glucose, or for osmotic control, with 15 mM mannitol (plus 5 mM glucose), and cultures were kept for 48 to 72 hours. The concentration of glucose in the control condition was 5 mM.

**Primary Culture of Rat Retinal Neural Cells**

All procedures involving animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinal cell cultures were obtained from 3- to 5-day-old Sprague-Dawley rats. Briefly, rat pups were anesthetized by intraperitoneal injection of ketamine (0.05 mL of 100 mg/mL) before death by rapid decapitation. Retinas were dissected in sterile Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-
1.2 NaCl, 4 KCl, 0.7 MgCl₂, 0.5 CaCl₂, 6 glucose, and 20 HEPES (pH 7.4) at 37°C, and then stimulated with 100 μM KA in the presence of 30 μM CTZ (to prevent AMPA receptor desensitization) and 5 mM CoCl₂, for 30 minutes. After stimulation, the cells were washed twice with HEPES buffer supplemented with 2 mM EDTA to remove excess extracellular Co²⁺. Intracellular Co²⁺ was precipitated with 0.12% Na₂S in HEPES buffer for 5 minutes. The cells were then washed with HEPES buffer and fixed with 4% paraformaldehyde containing 4% sucrose in PBS for 30 minutes at room temperature. CoS precipitates were enhanced by incubation in hot (50°C) developing buffer (in mM: 292 sucrose, 15.5 hydroquinone, and 42 citric acid), containing 1 mg/ml AgNO₃, for 1 hour in the dark. Staining was stopped by washing cells with hot developing buffer. To preserve the stain, we incubated the cells with 5% sodium thiosulfate at room temperature for 5 minutes. The cells were rinsed twice in PBS followed by washing with distilled water, air dried for 24 hours, and then mounted (Entellan; Merck). The preparations were visualized with a microscope (Axiovert 200; Carl Zeiss Meditec, Inc.) coupled to a photomicrograph system (Cool Snap HQ digital camera; Roper Scientific, Tucson, AZ). Images of five to seven arbitrary fields from each coverslip were photographed and the densitometry measurements were performed by delineating the cell bodies using the public-domain ImageJ program (http://rsb.info.nih.gov/ij/) developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Immunocytochemistry**

The cells were washed twice with PBS, fixed with 1% paraformaldehyde for 10 minutes at room temperature, rinsed twice in PBS, and permeabilized with 1% Triton X-100 in PBS for 5 minutes. After blocking for 1 hour with 10% donkey serum plus 0.1% Triton X-100 in PBS, the cells were incubated with the primary antibody (anti-MAP2, 1:100, or anti-GluR2, 1:250) for 90 minutes. The cells were rinsed three times with the blocking solution, incubated with a Cy2-conjugated secondary antibody (donkey anti-mouse IgG, 1:1000), were rinsed, and mounted (Aqua Poly/Mount; Polysciences, Inc., Warrington, PA). The preparations labeled with the anti-MAP2 antibody were visualized with a confocal microscope (488 nm excitation, 495–532 nm emission band; TCS SP2 AOBS; Leica, Deerfield, IL), and five random fields were photographed for each condition. The preparations labeled with anti-GluR2 antibody were visualized with a microscope (Axioshop 2 Plus; Carl Zeiss Meditec, Inc.), coupled to a camera (Axiocam HRc; Carl Zeiss Meditec., Inc.). For each condition at least five random fields were photographed and the densitometry measurements were performed by delineating the cell bodies using the public-domain ImageJ program (http://rsb.info.nih.gov/ij/) developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Live-Cell Confocal Measurement of Intracellular Calcium**

Primary cultures and R28 cells, plated on 35-mm glass-bottomed dishes, were loaded with 5 μM fluo-4/AM, for 20 minutes at 37°C, in saline buffer (in mM: 132 NaCl, 4 KCl, 1.4 MgCl₂, 1.4 CaCl₂, 6 glucose, and 10 HEPES [pH 7.4]). After they were rinsed the cells were placed on the stage of the inverted confocal microscope (63× oil immersion objective; TCS SP2 AOBS; Leica). Fluo-4 was excited with a 488-nm laser and emission was detected at 520 to 600 nm. Time-lapse images were acquired every 1.6 seconds. After 5 to 10 baseline images were acquired, the cells were stimulated with KCl or kainate. Image acquisition continued for 100 to 300 seconds. Image analysis was performed with the confocal microscope software. Briefly, regions of interest were defined by drawing an outline around each cell body, and the mean fluorescence was extracted across the time-lapse sequence of images to obtain fluorescence versus time plots for each cell. Background fluorescence was obtained from a region with no cells for every field examined and subtracted from the mean fluorescence. The mean fluorescence was also corrected for the mean baseline fluorescence determined before stimulation of the cells.

**Data Analysis**

Data are expressed as the mean ± SEM. Statistical significance was determined by analysis of variance (ANOVA), followed by the Dunnett post hoc test.

**RESULTS**

The effect of high glucose on the protein levels of AMPA (GluR1 and GluR2) and kainate (GluR6/7 and KA2) ionotropic glutamate receptor subunits was investigated, giving particular attention to the GluR2 subunit, because it controls the Ca²⁺ permeability of AMPA receptor-associated channels. The effect
of high glucose on changes in \([\text{Ca}^{2+}]_{\text{i}}\), evoked by membrane depolarization or AMPA receptor activation was also studied in primary cultures and R28 cells.

**Alteration of the Protein Levels of Ionotropic Glutamate Receptor Subunits**

The effect of high glucose on the expression of AMPA (GluR1 and GluR2) and kainate (GluR6/7 and KA2) receptor subunits was examined by immunoblot analysis of primary cell cultures from rat retina. In cells exposed to 30 mM glucose the protein content for the AMPA subunit, GluR1, was significantly less than control (67.3% \(\pm\) 4.6% of the control, Fig. 1A), whereas that of GluR2 was significantly greater than the control (225.5% \(\pm\) 49.1% of the control, Fig. 1B). The protein content of the GluR6/7 subunits was significantly less than the control (67.3% \(\pm\) 4.6% of the control, Fig. 1C). In contrast, the protein content of the KA2 subunit in cells exposed to 30 mM glucose was significantly greater than the control (204.3% \(\pm\) 42.3% of the control, Fig. 1D). The protein content of the glutamate receptor subunits in cells exposed to 25 mM mannitol (5 mM glucose), which was used as the osmotic control, were not significantly different from the control (5 mM glucose).

**Effect of High Glucose on \([\text{Ca}^{2+}]_{\text{i}}\) in Retinal Neurons**

Because high glucose increased the content of the GluR2 subunit, this may reduce the \([\text{Ca}^{2+}]_{\text{i}}\) permeability of the AMPA receptor-associated channel. The cobalt staining technique was used to assess the effect of high glucose on the uptake of \([\text{Ca}^{2+}]_{\text{i}}\) through AMPA receptor-associated channels.26,27 In control cultures (5 mM glucose), many cell bodies and processes were stained with cobalt (Fig. 2A). In contrast, cell cultures exposed to 30 mM glucose had fewer cells stained with cobalt, compared with the control (Fig. 2B). The number of cell bodies stained with cobalt was significantly reduced in glucose-treated cells compared with the control cells. (H) The length of processes stained with cobalt was significantly reduced in high-glucose-treated cells compared with the control. Results shown in (G) and (H) are expressed as the mean \(\pm\) SEM combined from results in four independent experiments. The images are representative of those obtained from four independent experiments performed in duplicate. **P< 0.01; one-way ANOVA followed by the Dunnett post hoc test. Bar, 40 \(\mu\text{m}.\)

**FIGURE 2.** High glucose levels reduced \([\text{Ca}^{2+}]_{\text{i}}\) uptake through AMPA receptors. Rat primary cell cultures were incubated in 5 mM glucose (Control), 30 mM glucose (Glucose), or 25 mM mannitol plus 5 mM glucose (Mannitol) for 7 days. The cells were stimulated with 100 \(\mu\text{M}\) KA under nondesensitizing conditions (with CTZ, 30 \(\mu\text{M}\)), in the presence of \([\text{Co}^{2+}].\) (A) In control cells, there was cobalt staining in both cell bodies and processes, (B) in high-glucose-treated cells there were fewer cell bodies and less cell processes stained with cobalt, (C) and in mannitol-treated cells cobalt staining was similar to that in control cells. (D–F) Cells were labeled for MAP2 by immunocytochemistry. There was no difference in the morphology of cells in any of the treatment groups. (G) The number of cell bodies stained with cobalt was significantly reduced in glucose-treated cells compared with the control cells. (H) The length of processes stained with cobalt was significantly reduced in high-glucose-treated cells compared with the control. Results shown in (G) and (H) are expressed as the mean \(\pm\) SEM combined from results in four independent experiments. The images are representative of those obtained from four independent experiments performed in duplicate. **P< 0.01; one-way ANOVA followed by the Dunnett post hoc test. Bar, 40 \(\mu\text{m}.\)
The presence of Ca\(^{2+}\)-permeable kainate receptor-associated channels was also investigated by Co\(^{2+}\) staining in cultured retinal neural cells. The selective activation of kainate receptors with 10 \(\mu\)M 4-methylglutamate or with 3 \(\mu\)M kainate did not result in Co\(^{2+}\) staining (data not shown). The failure of kainate receptor activation to induce Co\(^{2+}\) staining was not due to kainate receptor desensitization, because in the presence of 250 \(\mu\)g/mL concanavalin A, which inhibits kainate receptor desensitization, neither agonist caused Co\(^{2+}\) staining (data not shown). In addition, stimulation of cells with 100 \(\mu\)M kainate in the presence of LY 305070 (15 \(\mu\)M), which selectively blocks AMPA receptors, did not cause any Co\(^{2+}\) staining, indicating that staining was due to entry of Co\(^{2+}\) through Ca\(^{2+}\)-permeable AMPA receptors. These results indicate that the ion channel associated with AMPA receptors is less permeable to Ca\(^{2+}\) in high-glucose-treated cells, than the control cultures.

The decrease in cobalt uptake in high-glucose-treated cells may be due to an increased content of the GluR2 subunit. To examine this possibility, control cells and cells incubated with 30 mM glucose or 25 mM mannitol, were labeled by immunocytochemistry for the GluR2 subunit (Fig. 3). In control cells, GluR2 immunoreactivity was distributed throughout the cell bodies and processes (Fig. 3A). In most of the high-glucose-treated cells, the GluR2 immunoreactivity was enhanced, confirming that the GluR2 subunit was more abundant in these cells (Fig. 3B). GluR2 immunoreactivity in mannitol-treated cells was similar to control (Fig. 3C). Image analysis confirmed the significant increase in immunoreactivity in the high-glucose-treated cells compared with control and mannitol treatment (Fig. 3D).

**Effect of High Glucose on \([\text{Ca}^{2+}]_i\) Changes**

Calcium is a key mediator of many intracellular processes, and its deregulation can cause malfunction, leading to apoptosis.\(^{35}\) Voltage-sensitive \(\text{Ca}^{2+}\) channels are the most important pathway for the elevation of intracellular \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)), and their opening is dependent on the membrane potential. On activation, ionotropic glutamate receptors also contribute to the increase in \([\text{Ca}^{2+}]_i\). Therefore, the effect of high glucose on changes in \([\text{Ca}^{2+}]_i\), evoked by membrane depolarization or activation of AMPA and kainate receptors was studied by confocal microscopy on live cells loaded with the fluorescent membrane-permeable Ca\(^{2+}\) probe, fluo-4/AM. R28 cells were used as another model of retinal neurons. After stimulation with 20 mM KCl, the fluorescence intensity in control cells increased transiently and returned to baseline after 65 seconds (Figs. 4A, 4B). In cells grown in 20 mM glucose, the baseline fluorescence was similar to that in control cells (Fig. 4C). Membrane depolarization with KCl induced a significantly higher increase in the fluorescence, compared to control, and after 65 seconds the intensity in fluorescence was not similar to basal levels (Fig. 4D). Fluorescence intensity in high-glucose-treated cells did not recover to basal levels until the end of the experiment. As with control cells, in mannitol-treated cells the fluorescence was transiently increased by KCl and then returned to baseline (Fig. 4E). The effect of high glucose on the \([\text{Ca}^{2+}]_i\) changes evoked by either 20 mM KCl, kainate (100 \(\mu\)M) or kainate plus 50 \(\mu\)M CTZ (in Na\(^{+}\)-free medium), was also analyzed in primary cell cultures using fluo-4/AM. As observed in R28 cells, the fluorescence intensity evoked by 20 mM KCl in cells exposed to 30 mM glucose was significantly greater than in control cells (Fig. 5A). Moreover, high-glucose-treated cells did not recover to basal levels during the experiment. The fluorescence intensity in cells incubated with mannitol was not significantly different from that in control cells. Stimulation of primary cultures with 100 \(\mu\)M KA produced results similar to those obtained with KCl (Fig. 5B). The influx of Ca\(^{2+}\) through AMPA receptor-associated channels was examined by stimulating primary cultures with kainate in the presence of CTZ, which blocks desensitization of AMPA receptors, in a Na\(^{+}\)-free medium, in which Na\(^{+}\) ions were replaced by NMG. This molecule does not cross cell membranes and thus prevents membrane depolarization and
opening of voltage-sensitive Ca\textsuperscript{2+} channels. Also, Ca\textsuperscript{2+} does not enter through NMDA receptors, because the Mg\textsuperscript{2+} blockade is maintained under these conditions. Therefore, Ca\textsuperscript{2+} entry is exclusively due to the opening of AMPA receptors lacking the GluR2 subunit. Stimulation of primary cell cultures with 100 \mu M KA in the presence of 30 \mu M CTZ, in NMG medium, significantly increased fluorescence above baseline in control cells (Fig. 6). The fluorescence of cells exposed to high glucose was significantly decreased compared with control cells. This observation suggests that the AMPA receptors present in retinal neural cells incubated with high glucose were less permeable to Ca\textsuperscript{2+}. In mannitol-treated cells, the [Ca\textsuperscript{2+}]\textsubscript{i} changes evoked by 100 \mu M KA in the presence of 30 \mu M CTZ were similar to the control.

**DISCUSSION**

In this study, we found that elevated concentrations of glucose induce changes in the protein levels of several...
ionotropic glutamate receptor subunits and alter the Ca<sup>2+</sup> homeostasis in cultured retinal neural cells. These effects were not due to increases in osmolality, because equal concentrations of mannitol had no significant effect compared with the control.

AMPA and kainate receptors are important mediators of fast excitatory neurotransmission in the retina. In high-glucose-treated cell cultures, the protein content of GluR1 and GluR6/7 subunits decreased, whereas that of the GluR2 and KA2 receptor subunits increased. The expression of NMDA receptor subunits and the binding properties of AMPA receptors are also altered in the brain of diabetic rats, but the levels of GluR1 and GluR2/3 receptor subunits were unchanged.35–38 More recently, it was reported that the NR1 and GluR2/3 subunits are more abundant in the retina of diabetic rats.25 In our in vitro model, the changes in the content of ionotropic glutamate receptor subunits were not restricted to one type of receptor, since levels of both AMPA and kainate receptor subunits were altered by high glucose. The higher content of the GluR2 subunit is particularly significant because it decreases the Ca<sup>2+</sup>-permeability of AMPA receptor-associated channels.

Cobalt staining was used as a functional indicator of the Ca<sup>2+</sup>-permeability of the channel associated with AMPA receptors lacking the GluR2 subunit.39 Despite reports that kainate receptors may also mediate increases in intracellular Ca<sup>2+</sup>,35 in our hands, kainate receptors were not involved in the influx of cobalt. High glucose decreased the number of cells and the length of processes stained with cobalt, indicating a decrease in the number of Ca<sup>2+</sup>-permeable AMPA receptors, consistent with the increase in the protein content of the GluR2 subunit. Overall, the morphology of retinal neurons was not altered by high glucose, as observed by MAP2 immunoreactivity, suggesting that the decrease in the number of cell bodies and the length of the processes stained with cobalt in high-glucose-treated cells was not due to a widespread change in morphology but to a reduction in cobalt uptake.

On depolarization, voltage-sensitive Ca<sup>2+</sup> channels are the main route for Ca<sup>2+</sup> entry into the cells. In this study, the increase in [Ca<sup>2+</sup>], evoked by membrane depolarization or AMPA receptor activation was greater in high-glucose-treated cells than in control or mannitol-treated cells. This effect may be due to an increase in the abundance of voltage-sensitive Ca<sup>2+</sup> channels. Diabetes increases the density of Ca<sup>2+</sup> channels in other tissues (reviewed by Levy40). In dorsal root ganglion neurons from diabetic Bio Bred/Worchester (BB/W) rats there was an enhancement of high-voltage-activated currents, which increased with duration of diabetes up to 6 months.41 Similar results were found in skeletal muscle from 8-week STZ-diabetic rats.32 In addition, the [Ca<sup>2+</sup>], in cells treated with elevated concentrations of glucose did not recover to basal levels throughout the experiment, consistent with similar data from dorsal horn neurons of STZ-diabetic rats.25 The delay in recovery of [Ca<sup>2+</sup>], to baseline suggests that the mechanisms responsible for removing the [Ca<sup>2+</sup>], are also affected by high glucose.

N-methyl-D-glucamine (NMG) has been widely used as a Na<sup>+</sup> substitute to maintain physiological solutions as isotonic without Na<sup>+</sup>. This molecule does not enter through ion channels and therefore does not cause cell depolarization. In NMG medium, the voltage-sensitive Ca<sup>2+</sup> channels and NMDA receptors do not open, and Ca<sup>2+</sup> entry occurs essentially through AMPA receptors lacking the GluR2 subunit. Therefore, the influx of calcium specifically through AMPA receptors was examined by stimulating neural cells with kainate, in the presence of cyclothiazide to prevent desensitization of AMPA receptors. High glucose reduced the kainate-evoked [Ca<sup>2+</sup>], in NMG medium, compared with the control. This observation is consistent with the increase in the content of the GluR2 subunit and provides evidence that high glucose alters the function of AMPA receptors.

The combination of increased Ca<sup>2+</sup> influx and decreased Ca<sup>2+</sup> efflux can lead to sustained elevation in basal [Ca<sup>2+</sup>], which may trigger apoptosis.19 The increase in the abundance of the GluR2 AMPA subunit due to elevated glucose may therefore be a compensatory protective mechanism in response to intracellular Ca<sup>2+</sup> overload. The increase in the Ca<sup>2+</sup>-binding proteins calbindin and parvalbumin in the retina of diabetic rats is consistent with this possibility.25

Taken together, these results suggest that elevated glucose alters the content of ionotropic glutamate receptor subunits and Ca<sup>2+</sup> homeostasis in retinal neurons. This effect is likely to have an impact on normal function in the retina and may explain increased neuronal apoptosis and alterations in retinal function in diabetes. The alterations in Ca<sup>2+</sup> homeostasis found in retinal neurons may also play a role in the electroretinogram abnormalities induced by diabetes,43 as well as in the reduction in contrast and color sensitivity recorded in humans with diabetes.3–5

Acknowledgments

The authors thank Rhona Ellis for technical assistance with the confocal microscopy.

References


